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(71) Applicants: JOHN WAYNE CANCER INSTITUTE [US/US]; 2200 Santa Monica Boulevard, Santa Monica, CA 90404 (US). NATIONAL GENETICS INSTITUTE [US/US]; Suite 104, 5839 Green Valley Circle, Culver City, CA 90230 (US).

(72) Inventors: HOON, Dave, S., B.; 1911 Malcolm Avenue, No. 301, Los Angeles, CA 90025 (US). DOI, Fukashi; 1-1-13, Wakazono Kokum Minami-ku, Kitakyusyu 802 (JP). CONRAD, Andrew, J.; 687 Fifth Avenue, Los Angeles, CA 90049 (US). SCHMID, Peter; 10431 Regent Street, Los Angeles, CA 90034 (US).

(74) Agent: HIGHLANDER, Steven, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

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(54) Title: DETECTION OF MELANOMA OR BREAST METASTASES WITH A MULTIPLE MARKER ASSAY

(57) Abstract

A method for the diagnosis of melanoma or breast cancer is provided. In particular, the method provides for the detection of nucleic acids corresponding to multiple melanoma or breast cancer specific markers using template-dependent amplification processes. In one embodiment, the markers used are tyrosinase, MUC18, p97, MAGE-3, β-HCG, MAGE-1 and GalNAc. The methods using these combinations of markers are more sensitive in the detection of tumor cells in patients as compared to single marker assays.

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DESCRIPTION

DETECTION OF MELANOMA OR BREAST METASTASES WITH A MULTIPLE MARKER ASSAY

BACKGROUND OF THE INVENTION

Some of the work described in this application was supported by grant number PO1 CA1038 from the National Cancer Institute.

1. Field of the Invention

The present invention relates generally to the field of cancer diagnostic techniques. In particular, the invention relates to the detection of genetic markers indicative of melanoma or breast cancer cells. In one example, detection of multiple markers is achieved by polymerase chain reaction assay.

2. Description of the Related Art

Cancers are one of the leading causes of disease, being responsible for 526,000 deaths in the United States each year (Boring *et al.*, 1993). For example, breast cancer is the most common form of malignant disease among women in Western countries and, in the United States, is the most common cause of death among women between 40 and 55 years of age (Forrest, 1990). The incidence of breast cancer is increasing, especially in older women, but the cause of this increase is unknown. Malignant melanoma is another form of cancer whose incidence is increasing at a frightening rate, at least sixfold in the United States since 1945, and is the single most deadly of all skin diseases (Fitzpatrick, 1986).

One of the most devastating aspects of cancer is the propensity of cells from malignant neoplasms to disseminate from their primary site to distant organs and develop into metastases. Despite advances in surgical treatment of primary neoplasms and aggressive therapies, most cancer patients die as a result of metastatic disease. Animal tests indicate that about 0.01% of circulating cancer cells from solid tumors establish successful metastatic colonies (Fidler, 1993).

Thus, the detection of occult cancer cells in circulation is important in assessing the level of tumor progression and metastasis. Because subclinical metastasis can remain dormant for many years, monitoring of patients' blood for circulating tumor cells may prove advantageous in detecting tumor progression before metastasis to other organs occurs. Assessment of circulating tumor cells also would provide a rapid monitoring system to determine if a specific therapy is effective.

For example, recognition of metastases in tumor-draining lymph nodes (TDLN) now has been shown critical for patient management. It is known that between 25-30 per cent of breast cancer patients with node negative, localized disease will relapse within five years after operative intervention (Henderson *et al.*, 1989). Accurate axillary staging of TDLN in detection of metastases has been an important factor for selecting patients for adjuvant therapy (NIH, 1992; Giuliano, *et al.*, 1995; Giuliano, *et al.*, 1994). Several retrospective studies on breast cancer TDLN demonstrated that analysis of multiple sections of nodes shown to be tumor negative were found to have occult metastases (Bettelheim, *et al.*, 1990; Chen *et al.*, 1991; Neville *et al.*, 1991). The identification of nodes with occult metastases were shown to significantly correlate to

poorer prognosis (Bettelheim *et al.*, 1990; Neville *et al.*, 1991).

Previous tumor diagnostic techniques have focused on the detection of tumor associated antigens or on molecules released by tumor cells (Smart, 1990; Moertel *et al.*, 1993; Stamey *et al.*, 1989). At best, these assays only detect tumors with no indication of metastatic potential or tumor progression. In addition, such assays measure a single antigen whose release is often proportional to the size of the tumor and they cannot account for heterogeneity of individual markers in tumor lesions, both within individual patients or among patient groups.

The recent development of the PCR assay (Mullis and Faloona, 1987; Erlich, 1989) for detection of occult metastatic tumor cells in blood using specific markers has provided a new approach to assess tumor progression (Smith *et al.*, 1991; Naito *et al.*, 1991). In one study, circulating melanoma cells in blood were detected by PCR analysis using the tyrosinase gene marker (Smith *et al.*, 1991). Seven melanoma patients with metastatic disease were analyzed, but only four were positive. Other studies using PCR have been used to detect circulating tumor cells in melanoma, as well as in breast, prostate and neuroblastoma cancer patients (Smith *et al.*, 1991; Datta *et al.*, 1994; Moreno *et al.*, 1992; Naito *et al.*, 1991). These studies, employing a single marker, were limited by their ability to discriminate cancer cells from normal cells also carrying the marker, thus reducing specificity and reliability. In addition, tumor heterogeneity has caused sensitivity problems where a single, specific marker has been employed.

As indicated above, tumors are notoriously heterogeneous, particularly in advanced stages of tumor

progression (Morton *et al.*, 1993; Fidler and Hart, 1982; Nowell, 1982; Elder *et al.*, 1989; Bystryn *et al.*, 1985). Although tumor cells within a primary tumor or metastasis all may express the same marker gene, the level of specific mRNA expression can vary considerably (Elder *et al.*, 1989). It is, therefore, necessary to develop a detection system that can cope with such heterogeneous targets.

Thus, despite the identification of melanoma and breast cancer markers, these markers cannot individually detect tumor cells in a highly specific and sensitive manner. This is due to the wide phenotypic diversity found in tumor cells at any one time and during disease differentiation. There remains a need to develop a more sophisticated approach, that can accommodate such a biological heterogeneous situation in order to sensitively and specifically detect metastasis and diagnosis disease stage.

3. Summary of the Invention

The present invention seeks to overcome these and other drawbacks inherent in the prior art by providing sensitive and accurate methods for the detection of melanoma or breast cancer cells in a biological sample. The methods provide for the detection of melanoma or breast cancer cells in a biological sample by amplifying at least two nucleic acids from the sample, the nucleic acids being markers for melanoma or breast cancer cells.

The present invention comprises the following steps. A nucleic acid is extracted from a biological sample. The nucleic acid is contacted with a first primer pair that hybridizes to a first melanoma or breast cancer marker nucleic acid. The primers are extended by polymerase to produce an amplification product. This

process is repeated a sufficient number of times to permit detection of the amplification product. Finally, all steps are repeated with the same biological sample and a second primer pair that hybridizes to another melanoma or breast cancer marker nucleic acid.

In preferred embodiments, the method may further comprise preparing at least two pairs of primers complementary to regions of melanoma or breast cancer marker nucleic acids. In another embodiment, the method may further comprise of preparing primer pairs for at least three, four, five, six or even seven melanoma or breast cancer markers.

In preferred embodiments of the invention, the markers amplified and detected are selected from the group comprising tyrosinase, MAGE-3, MUC18, p97, MAGE-1, GalNAc and β-HCG. The preferred method of amplification is by reverse transcription and polymerase chain reaction (PCR). In one embodiment of the invention the PCR further comprises nested PCR.

In one embodiment, the nucleic acid is RNA. Preferably, the RNA extracted from a biological sample is total cellular RNA. In a preferred embodiment, the total cellular RNA is converted to DNA prior to amplification.

In certain embodiments of the invention, the biological sample is a body tissue or body fluid. In preferred embodiments, the body tissue is bone marrow aspirate, bone marrow biopsy, lymph node aspirate, lymph node biopsy, spleen tissue, fine needle aspirate, skin biopsy or organ tissue biopsy. Other embodiments include samples where the body fluid is peripheral blood, lymph fluid, ascites, serous fluid, pleural effusion, sputum, cerebrospinal fluid, lacrimal fluid, stool or

urine. In a preferred embodiment, the biological sample is of human origin.

In preferred embodiments of the invention, the method includes separation of the amplification product by gel electrophoresis. In other embodiments, the method of separation is by chromatographic techniques. In a preferred embodiment of the invention, hybridization with a labeled probe permits identification of the amplification product following separation.

In further embodiments, the present invention encompasses a kit for use in detecting melanoma or breast cancer cells in a biological sample comprising, pairs of primers for amplifying nucleic acids corresponding to the marker genes, and containers for each of these primers. In preferred embodiments, the kit further comprises enzymes and reagents for the preparation of cDNA's and amplification thereof. In yet more preferred embodiments, the kit further comprises enzymes and reagents for radiochemical or chromophoric labeling of nucleic acids.

4. Detailed Description of the Preferred Embodiments

The present invention pertains to a sensitive, multimarker assay to detect occult melanoma or breast cancer cells in the blood of patients with or without clinical evidence of disease. This assay is designed to overcome limitations in existing technologies with respect to both sensitivity and specificity.

In its most general form, the instant invention comprises a method for identification of melanoma or breast cancer cells in a biological sample by amplifying and detecting nucleic acids corresponding to melanoma or breast cancer cell markers. The biological sample can be

any tissue or fluid in which melanoma or breast cancer cells might be present. Preferred embodiments include bone marrow aspirate, bone marrow biopsy, lymph node aspirate, lymph node biopsy, spleen tissue, fine needle aspirate, skin biopsy or organ tissue biopsy. Other embodiments include samples where the body fluid is peripheral blood, lymph fluid, ascites, serous fluid, pleural effusion, sputum, cerebrospinal fluid, lacrimal fluid, stool or urine.

Nucleic acid used as template for amplification is isolated from cells contained in the biological sample according to standard methodologies. (Sambrook *et al.*, 1989) The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary cDNA. In a preferred embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

Pairs of primers that selectively hybridize to genes corresponding to specific markers are contacted with the isolated nucleic acid under conditions that permit selective hybridization. Once hybridized, the nucleic acid:primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

Next, the amplification product is detected. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even via

a system using electrical or thermal impulse signals (Affymax technology, Bellus, 1994).

The foregoing process is conducted at least twice on a given sample using at least two different primer pairs specific for two different specific markers. Following detection, one may compare the results seen in a given patient with a statistically significant reference group of normal patients and melanoma or breast cancer patients. In this way, it is possible to correlate the number and kind of markers with various clinical states.

(i) Melanoma-Specific or Breast Cancer-Specific Markers

While the present invention exemplifies several markers, any marker that is correlated with the presence or absence of melanomas or breast cancer may be used. A marker, as used herein, is any proteinaceous molecule (or corresponding gene) whose production or lack of production is characteristic of a melanoma or breast cancer cell. Depending on the particular set of markers employed in a given analysis, the statistical analysis will vary. For example, where a particular combination of markers is highly specific for melanomas or breast cancer, the statistical significance of a positive result will be high. It may be, however, that such specificity is achieved at the cost of sensitivity, *i.e.*, a negative result may occur even in the presence of melanoma or breast cancer. By the same token, a different combination may be very sensitive, *i.e.*, few false negatives, but has a lower specificity.

As new markers are identified, different combinations may be developed that show optimal function with different ethnic groups or sex, different geographic distributions, different stages of disease, different

degrees of specificity or different degrees of sensitivity. Marker combinations may also be developed, which are particularly sensitive to the effect of therapeutic regimens on disease progression. Patients may be monitored after surgery, hyperthermia, immunotherapy, cytokine therapy, gene therapy, radiotherapy or chemotherapy, to determine if a specific therapy is effective.

One particularly useful combination of markers is tyrosinase and p97. Human tyrosinase is an essential enzyme which regulates the production of melanin (Nordlund *et al.*, 1989; Hoon *et al.*, 1993), a group of brown or black pigments in the skin and eyes of humans. More specifically, tyrosinase catalyzes the conversion of tyrosine to Dopa and of Dopa to dopaquinone. p97, also known as melanotransferrin, is a cell surface sialoglycoprotein that bears some sequence homology to transferrin (Brown *et al.*, 1981; Rose *et al.*, 1986). Like transferrin, p97 binds iron, thereby being implicated in iron metabolism.

There are many other markers that may be used in combination with these, and other, markers. For example, β-human chorionic gonadotropin (β-HCG). β-HCG is produced by trophoblastic cells of placenta of pregnant woman and is essential for maintenance of pregnancy at the early stages (Pierce *et al.*, 1981; Talmadge *et al.*, 1984). β-HCG is known to be produced by trophoblastic or germ cell origin tumors, such as choriocarcinoma or testicular carcinoma cells (Madersbacher *et al.*, 1994; Cole *et al.*, 1983). Also ectopic expression of β-HCG has been detected by a number of different immunoassays in various tumors of non-gonadal such as breast, lung, gastric, colon, and pancreas, etc. (McManus *et al.*, 1976; Yoshimura *et al.*, 1994; Yamaguchi *et al.*, 1989; Marcillac *et al.*, 1992; Alfthan *et al.*, 1992). Although the

function of β-HCG production in these tumors is still unknown, the atavistic expression of β-HCG by cancer cells and not by normal cells of non-gonadal origin suggests it may be a potentially good marker in the detection of melanoma and breast cancer (Tormey *et al.*, 1977; Tormey *et al.*, 1975).

Another exemplary example of a marker is glycosyltransferase β-1, 4-N-acetylgalacto-saminyltransferase (GalNAc). GalNAc catalyzes the transfer of N-acetylgalactosamine by β1,4 linkage onto both gangliosides GM3 and GD3 to generate GM2 and GD2, respectively (Nagata, *et al.*, 1992; Furukawa *et al.*, 1993). It also catalyzes the transfer of N-acetylgalactosamine to other carbohydrate molecules such as mucins. Gangliosides are glycosphingolipids containing sialic acids which play an important role in cell differentiation, adhesion and malignant transformation. In melanoma, gangliosides GM2 and GD2 expression, are often enhanced to very high levels and associated with tumor progression including metastatic tumors (Hoon *et al.*, 1989; Ando *et al.*, 1987; Carubia *et al.*, 1984; Tsuchida *et al.*, 1987a). Gangliosides are also highly expressed in breast cancer cells. The gangliosides GM2 and GD2 are immunogenic in humans and can be used as a target for specific immunotherapy such as human monoclonal antibodies or cancer vaccines (Tsuchida *et al.*, 1987b; Irie, 1985).

GalNAc mRNA may be used as a marker of GM2 and GD2 expression and consequently a marker of either melanoma or breast cancer cells. GalNAc is generally not expressed in normal lymphocytes, epithelial cells, melanocytes, connective tisue or lymph node cells. If detected, it is in very low levels.

Other markers contemplated by the present invention include cytolytic T lymphocyte (CTL) targets. MAGE-3 is a marker identified in melanoma cells and breast carcinoma. MAGE-3 is expressed in many melanomas as well as other tumors and is a (CTL) target (Gaugler *et al.*, 1994). MAGE-1 and MAGE-2 are other members of the MAGE gene family. MAGE-1 gene sequence shows 73% identity with MAGE-3 and expresses an antigen also recognized by CTL (Gaugler *et al.*, 1994). MART-1 is another potential CTL target (Robbins *et al.*, 1994) and may also be included in the present invention.

MUC18 is another marker that is useful in the identification of melanoma cells (Lehmann *et al.*, 1989; Lehmann *et al.*, 1987). MUC18 is a cell surface glycoprotein that is a member of the immunoglobulin superfamily and possesses sequence homology to neural cell adhesion molecules (NCAM). Other mucin family members include MUC1, MUC2, MUC3 and MUC4. These were found to be expressed at a high level in certain tumor cell lines (Hollingsworth *et al.*, 1994) and may also be used as markers in the present invention.

Other members of the immunoglobulin superfamily of adhesions molecules associated with the development of melanoma metastasis (Denton *et al.*, 1992) may be utilized in the present invention. Preferred examples include intercellular adhesion molecule-1 (ICAM-1), NCAM, VCAM-1, and ELAM. Another preferred embodiment of the invention, includes cell adhesion molecules associated with other metastatic diseases, such as carcinoembryonic antigen (CEA) and DCC (deleted in colorectal cancer) (Johnson, 1991).

Other breast or skin cancer associated proteins and their corresponding nucleic acids may also be utilized in the present invention. Preferred examples include

melanoma antigen gp75 (Vijayasardahi *et al.*, 1990), human cytokeratin 8 (HKer 8) (Pittman *et al.*, 1993), high molecular weight melanoma antigen (Natali *et al.*, 1987) and Keratin 19 (K19) (Datta *et al.*, 1994). This list is not intended to be exhaustive, but merely exemplary, for the type and number of potential markers which may be used in the present invention.

Other proteins and their corresponding nucleic acids related to the melanin synthesis pathway may be used as markers, such as tyrosinase related protein 1 and 2 and members of the pMel 17 gene family (Kwon *et al.*, 1993).

Preferred embodiments of the invention involve many different combinations of markers for the detection of melanoma breast cancer cells. Any marker that is indicative of neoplasia in breast cells or melanocytes may be included in this invention. However, preferred embodiments include combinations of tyrosinase, MAGE-3, MUC18, p97, β-HCG, GalNAc and MAGE-1. Table 1, as disclosed herein, represent partially useful combinations of markers which may be employed for the detection of melanoma or breast cancer cells.

Table 1. Preferred Multiple Marker Combinations

Table 1A. Combinations of Six or Seven Multiple Markers.

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
+	+	+	+	+	+	+
+	+	+	+	+	+	-
+	+	+	+	+	-	+
+	+	+	+	-	+	+
+	+	+	-	+	+	+
+	+	-	+	+	+	+
+	-	+	+	+	+	+
-	+	+	+	+	+	+

Table 1B. Combinations of Five Multiple Markers

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
+	+	+	+	+	-	-
+	+	+	+	-	-	+
+	+	+	-	-	+	+
+	+	-	-	+	+	+
+	-	-	+	+	+	+
-	-	+	+	+	+	+
-	+	+	+	+	+	-
+	+	+	+	-	+	-
+	+	+	-	+	-	+
+	+	-	+	-	+	+
+	-	+	-	+	+	+
-	+	-	+	+	+	+
+	-	+	+	+	+	-
-	+	+	+	+	-	+
+	+	+	-	+	+	-
+	+	-	+	+	-	+
+	-	+	+	-	+	+
-	+	+	-	+	+	+
+	+	-	+	+	+	-
+	-	+	+	+	-	+
-	+	+	+	-	+	+

Table 1C. Combinations of Four Multiple Markers

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
+	+	+	+	-	-	-
+	+	+	-	-	-	+
+	+	-	-	-	+	+
+	-	-	-	+	+	+
-	-	-	+	+	+	+
-	-	+	+	+	+	-
-	+	+	+	+	-	-
+	+	+	-	-	+	-
+	+	-	-	+	-	+
+	-	-	+	-	+	+
-	-	+	-	+	+	+
-	+	-	+	+	+	-
+	-	+	+	+	-	-
-	+	+	+	-	-	+
+	+	+	-	+	-	-
+	+	-	+	-	-	+
+	-	+	-	-	+	+
-	+	-	-	+	+	+
+	-	-	+	+	+	-
-	-	+	+	+	-	-
-	+	+	+	-	+	-
+	+	-	-	+	+	-
+	-	-	+	+	-	+
-	-	+	+	-	+	+
-	+	+	-	+	+	-
+	+	-	+	+	-	-
+	-	+	+	-	-	+
-	-	+	-	-	+	+

Table 1D. Combinations of Three Multiple Markers

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
-	-	-	-	+	+	+
-	-	-	+	+	+	-
-	-	+	+	+	-	-
-	+	+	+	-	-	-
+	+	+	-	-	-	-
+	+	-	-	-	-	+
+	-	-	-	-	+	+
-	-	-	+	+	-	+
-	-	+	+	-	+	-
-	+	+	-	+	-	-
+	+	-	+	-	-	-
+	-	+	-	-	-	+
-	+	-	-	-	+	+
+	-	-	-	+	+	-
-	-	-	+	-	+	+
-	-	+	-	+	+	-
-	+	-	+	+	-	-
+	-	+	+	-	-	-
-	+	+	-	-	-	+
+	+	-	-	-	+	+
+	-	-	-	+	-	+
-	-	+	+	-	-	+
-	+	+	-	-	+	-
+	+	-	-	+	-	-
+	-	-	+	-	-	+
-	-	+	-	-	+	+
-	+	-	-	+	+	-
+	+	-	+	+	-	-

Table 1E. Combinations of Two Multiple Markers

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
-	-	-	-	-	+	+
-	-	-	-	+	+	-
-	-	-	+	+	-	-
-	-	+	+	-	-	-
-	+	+	-	-	-	-
+	+	-	-	-	-	-
+	-	-	-	-	-	+
-	-	-	-	+	-	+
-	-	-	+	-	+	-
-	-	+	-	+	-	-
-	+	-	+	-	-	-
+	-	+	-	-	-	-
-	+	-	-	-	-	+
+	-	-	-	-	+	-
-	-	-	+	-	-	+
-	-	+	-	-	+	-
-	+	-	-	+	-	-
+	-	-	+	-	-	-
-	-	+	-	-	-	+
-	+	-	-	-	+	-
+	-	-	-	+	-	-

+ markers included in the combination; - markers not included.

(ii) Primers

The term primer, as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be

provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

In most cases, it will be preferable to synthesize desired oligonucleotides. Suitable primers can be synthesized using commercial synthesizers, such as those supplied by Applied Biosystems (Foster City, CA) using methods well known to those of ordinary skill in the art. Where double-stranded primers are desired, synthesis of complementary primers is performed separately and the primers mixed under conditions permitting their hybridization.

Selection of primers is based on a variety of different factors, depending on the method of amplification and the specific marker involved. For example, the choice of primer will determine the specificity of the amplification reaction. The primer needs to be sufficiently long to specifically hybridize to the marker nucleic acid and allow synthesis of amplification products in the presence of the polymerization agent and under appropriate temperature conditions. Shorter primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the marker nucleic acid and may be more susceptible to non-specific hybridization and amplification.

Primer sequences do not need to correspond exactly to the specific marker sequence. Non-complementary nucleotide fragments may be attached to the 5' end of the primer with the remainder of the primer sequence being complementary to the template. Alternatively, non-complementary bases can be interspersed into the primer, provided that the primer sequence has sufficient complementarily, in particular at the 3' end, with the

template for annealing to occur and allow synthesis of a complementary DNA strand.

In preferred embodiments, primers may be designed to hybridize to specific regions of the marker nucleic acid sequence. For example, GC rich regions are favored as they form stronger hybridization complexes than AT rich regions. In another example, primers are designed, solely, to hybridize to a pair of exon sequences, with at least one intron in between. This allows for the activity of a marker gene to be detected as opposed to its presence by minimizing background amplification of the genomic sequences and readily distinguishes the target amplification by size.

Primers also may be designed to amplify a particular segment of marker nucleic acid that encodes restriction sites. A restriction site in the final amplification product would enable digestion at that particular site by the relevant restriction enzyme to produce two products of a specific size. Any restriction enzyme may be utilized in this aspect. This added refinement to the amplification process may be necessary when amplifying a marker nucleic acid sequence with close sequence similarity to other nucleic acids. Alternatively, it may be used as an added confirmation of the specificity of the amplification product.

(iii) Template Dependent Amplification Methods

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCR) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1990, each of which is incorporated herein

by reference in its entirety. Briefly, in PCR, two primer sequences are prepared which are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated. Preferably a reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternatively, preferred methods for reverse transcription utilize thermostable DNA polymerases. These methods are described in WO 90/07641 filed December 21, 1990. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPO No. 320 308, incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the marker sequence, each pair will bind to opposite complementary strands of the marker such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR, bound ligated units dissociate from the marker and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent No. 4,883,750 describes a method similar to LCR for binding probe pairs to a marker sequence.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA which has a region complementary to that of a marker is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence which can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of marker molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention. Walker *et al.*, 1992, incorporated herein by reference in its entirety.

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR) involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Marker specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having a 3' and 5' sequences of non-specific DNA and middle sequence of specific RNA is hybridized to DNA which is present in a sample. Upon hybridization, the reaction is treated with RNaseH, and the products of the probe identified as distinctive products which are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in British Patent Application No. 2,202,328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR like, template and enzyme dependent synthesis. The primers may be modified by labelling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the marker sequence, the probe binds and is cleaved catalytically. After cleavage, the marker sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the marker sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR. Kwoh *et al.*, 1989; Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference in their entirety. In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has marker specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second marker specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by a polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNAs are

reverse transcribed into double-stranded DNA, and transcribed once against with a polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate marker specific sequences.

Davey *et al.*, EPO No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a first template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a second template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting as a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme

based on the hybridization of a promoter/primer sequence to a marker single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "race" and "one-sided PCR." Frohman, M.A., 1990 and Ohara *et al.*, 1989, each incorporated herein by reference in their entirety.

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention. Wu *et al.*, 1989, incorporated herein by reference in its entirety.

(iv) Separation Methods

Following amplification, it may be desirable to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification occurred. In a preferred embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods. See Sambrook *et al.*, 1989. In a preferred embodiment, the gel is a 2% agarose gel.

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography (Freifelder, 1982).

(v) Identification Methods

Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

Alternatively, separation may be unnecessary. These methods may be collectively termed Sequencing By Hybridization or SBH (Cantor *et al.*, 1992; Drmanac & Crkvenjakov, U.S. Patent No. 5,202,231). Development of certain of these methods has given rise to new solid support type sequencing tools known as sequencing chips. The utility of SBH in general is evidenced by the fact that U.S. Patents have been granted on this technology.

SBH can be conducted in two basic ways, often referred to as Format 1 and Format 2 (Cantor *et al.*, 1992). In Format 1, oligonucleotides of unknown sequence, generally of about 100-1000 nucleotides in length, are arrayed on a solid support or filter so that the unknown samples themselves are immobilized (Strezoska *et al.*, 1991; Drmanac & Crkvenjakov, U.S. Patent No. 5,202,231). Replicas of the array are then interrogated by hybridization with sets of labeled probes of about 6 to 8 residues in length.

In Format 2, a sequencing chip is formed from an array of oligonucleotides with known sequences of about 6 to 8 residues in length (Southern, WO 89/10977; Khrapko *et al.*, 1991; Southern *et al.*, 1992). The nucleic acids of unknown sequence are then labeled and allowed to

hybridize to the immobilized oligos. In another embodiment, hybridization may be detected by electrical or thermal impulse signals (Affymax Technology, Bellus, 1994).

In a preferred method, however, visualization is achieved indirectly. Following separation of amplification products, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, where the other member of the binding pair carries a detectable moiety.

In a particularly preferred embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989. Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with a chromophore conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U.S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to

carrying out methods according to the present invention.

(vi) Clinical Stages of Malignant Melanoma

Cancers are staged according to a well-defined, elaborate progressive scale, developed by the American Joint Committee on Cancer.

Malignant melanomas can arise in any skin area that contains melanocytes, but body moles, also called pigmented nevi, are particularly vulnerable. Although some moles, especially those on the face and torso, originate in pigment cells, they sometimes contain little pigment and are light in color. All moles are initially benign tumors of varying shape, but it is significant to note that about 20 to 30 percent of all melanomas begin in the pigment cells of moles.

Caught early, melanoma is very often curable. On the other hand, melanomas that are not detected until they have invaded even a few millimeters of the deeper layers of skin have a much poorer prognosis. The five-year survival rate varies considerably depending on stage level. For Stage I and Stage II melanoma, the five-year survival rate is over 80%. However, for Stage IV the survival rate is less that 20% (AJCC, .

A simplified summary of the scale, developed by the American Joint Committee for the Staging of melanoma is presented in Table 2.

Table 2: Staging of Melanoma

Stage I: Primary site, small tumor Negative lymph nodes No detectable metastases
Stage II: Invasion beyound primary site Lymph nodes negative may have one positive No detectable distant metastases
Stage III: Tumors at regional skin or subcutaneous sites, primarily located to lymph nodes.
Stage IV: Tumor of any size Lymph nodes either positive or negative Distant metastases to multiple sites

Metastasis to a distal organ may or may not result in secondary metastasis to other organs. Since subclinical metastasis can remain dormant for many years, monitoring of a patient's blood for circulating tumor cells may be helpful in detecting tumor progression before clinically evident metastases to other organs are detected.

(vii) Clinical Stages of Breast Cancer

Many factors appear to influence the chances of surviving breast cancer. Early detection and treatment are the most important. The overall five-year survival rate is about 75 percent for white women and about 63 percent for black women. This rises to nearly 90 percent for women with Stage I or II cancer that is treated while the cancer is confined to the breast (Scanlon and Strax, 1986).

A simplified summary of the scale, developed by the American Joint Committee for the Staging of Breast Cancer in 1982, is presented in Table 3.

Table 3: Staging of Breast Cancers

Stage I: Small tumor (less than 2 cm or .78 inches) Negative lymph nodes No detectable metastases
Stage II: Tumor greater than 2 cm but less than 5 cm Lymph nodes negative or Tumor less than 5 cm across Lymph nodes positive No detectable distant metastases
Stage III: Large tumor (greater than 5 cm) or Tumor of any size with invasion of skin or chest wall or "grave signs" or Associated with positive lymph nodes in the collarbone area but No detectable distant metastases
Stage IV: Tumor of any size Lymph nodes either positive or negative Distant metastases

(viii) Kit Components

All the basic essential materials and reagents required for detecting melanoma or breast cancer cells in a biological sample, may be assembled together in a kit. This will generally comprise of the preselected primers for two, or more, particular specific markers. Also included may be enzymes suitable for amplifying nucleic acids including various polymerases (RT, Tag, etc.), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification.

Such kits will generally comprise, in suitable means, distinct containers for each individual reagent and enzyme as well as for each marker primer pair. Preferred pairs of primers for amplifying nucleic acids correspond to the genes for tyrosinase, MAGE-3, MUC18, p97, MAGE-1, GalNAc and β-HCG.

Kits of the present invention, also will typically include a means for containing the reagents in close confinement for commercial sale such as, *e.g.*, injection or blow-molded plastic containers into which the desired reagent are retained. Other containers suitable for conducting certain steps of the disclosed methods also may be provided.

5. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I

Detection of Multiple Marker RNA Expression in Melanoma Cells

A. MATERIALS AND METHODS

(i) Melanoma cell lines

Melanoma cell lines M10, M12, M24, M101, Mke, Mst, Mmu, Mka, and Mkn were established and characterized at the John Wayne Cancer Institute (JWCI). Cells were grown in RPMI 1640 plus 10% fetal calf serum (heat-inactivated) (Gemini, Calabasas, CA) plus penicillin and streptomycin

(GIBCO, Long Island, NY) in T75 cm^2 flasks. Adherent cell lines were routinely passaged by trypsinization every 3-4 days (Hoon *et al.*, 1993). For PCR studies, cell lines were used when 75-85% confluent.

(ii) Blood preparation and RNA extraction

Peripheral blood lymphocytes (PBL) were obtained from the buffy coat of 15 ml of blood from healthy normal donors. The cells were washed by centrifugation for 5 min.

Total cellular RNA was extracted using the UltraSpec isolation system (Biotecx, Houston, TX) or Tri-Reagent isolation system (Molecular Research Center, Inc., Cincinnati OH) as described by the manufacturer. For UltraSpec, the cells were lysed in 2 ml of UltraSpec RNA reagent by repetitive pipetting and placed in ice for 5 min. Four hundred μl of chloroform was added and mixed rigorously for 15 sec and placed on ice for 5 min. The solution was centrifuged at 12,000 X g at 4°C for 15 min. The upper phase was transferred into a RNAse-free eppendorf tube, 1 volume of isopropanol was added and the solution was precipitated at 4°C for 10 min. The tube was centrifuged at 12,000 g at 4°C for 20 min. The sample was washed with 70% ethanol, dried, and resuspended in 50 μl of DEPC (diethylpyrocarbonate)-treated Tris-EDTA (TE) buffer.

For Tri-Reagent, the cells were lysed in 1 ml of Tri-Reagent by repetitive pipetting and were placed on ice for 5 min. Two hundred μl of chloroform was added and mixed vigorously for 15 sec and incubated at room temperature for 5 min. The solution was then centrifuged at 12,000 X g at 4°C for 15 min. The upper aqueous phase was transferred into an RNAse-free eppendorf tube, equal volume of isopropanol was then added and the nucleic acid

was allowed to precipitate at room temperature for 10 min. The tube was then centrifuged as 12,000 X g at 4°C for 10 min. The sample was washed twice with 70% ethanol, vacuum-dried, and resuspended in 10mM Tris-HCl with 1 ml EDTA solution (pH 7.4). The concentration of total RNA was determined using a Beckman spectrophotometer. One μg of total RNA was used in the PCR assay to detect mRNA.

All extraction procedures for each specimen were carried out separately in a designated laminar flow hood under sterile conditions to avoid potential RNA cross-contamination. PCR reagent set up and post-PCR gel electrophoresis were carried out in separate rooms to avoid potential RNA cross-contamination.

(iii) Oligonucleotide primers and probes

Oligonucleotide primers were synthesized and purified at the Molecular Biology Institute Core Facility, UCLA. Oligonucleotide 5-' and 3'-primers for individual genes were as follows: MAGE-3 primers were 5'-GAAGCCGGCCCAGGCTCG-3' (SEQ ID NO: 1) and 5'-GGAGTCCTCATAGGATTGGCTCC-3'(SEQ ID NO: 2); MUC18 primers were 5'-CCAAGGCAACCTCAGCCATGTC-3' (SEQ ID NO: 3) and 5'-CTCGACTCCACAGTCTGGGACGACT-3'(SEQ ID NO: 4); MUC18 nested primers were 5'-GTCATCTTCCGTGTGCGCCA-3'(SEQ ID NO: 5) and 5'-GTAGCGACCTCCTCAGGCTCCTTAC-3'(SEQ ID NO: 6); tyrosinase were 5'-TTGGCAGATTGTCTGTAGCC-3' (SEQ ID NO: 7) and 5'-AGGCATTGTGCATGCTGCTT-3'(SEQ ID NO: 8); tyrosinase nested primers were 5'-GTCTTTATGCAATGGAACGC-3' (SEQ ID NO: 9) and 5'-GCTATCCCAGTAAGTGGACT-3'(SEQ ID NO: 10); and p97 primers were 5'-TACCTGGTGGAGAGCGGCCGCCTC-3' (SEQ ID NO: 11) and 5'-AGCGTCTTCCCCATCAGTGT-3' (SEQ ID NO: 12). The amplification products of MAGE-3, MUC18, MUC18 "nested," tyrosinase, tyrosinase "nested" and p97 were 423, 437, 262, 284, 207 and 286 bp, respectively.

(iv) Reverse Transcription and Polymerase chain reaction (RT-PCR)

The RT-PCR assay was performed as previously described with some modifications (Hoon *et al.*, 1993). An oligo $(dT)_{15}$ primer was employed for the reverse transcription step, to produce cDNA and guarantee its amplification over genomic DNA. The reverse transcription (RT) mixture consisted of 4 μl 25 mM $MgCl_2$, 2 μl 10X RT buffer, 4 μl 10 mM dinucleotide triphosphate mixture, 0.5 μl RNAs in (40 U/μl), 1 μl AMV reverse transcriptase (9 U/μl), and 1 μl oligo$(dT)_{15}$ primer (1.5 μg/μl). Three μg of sample RNA was added to the RT mixture and H_2O was added to bring the volume up to 20 μl. All reagents were obtained from Promega (Madison, WI). The reaction was incubated at 42°C for 2 hr, 99°C for 5 min, and on ice for 5 min.

The PCR mixture consisted of 10 μl 10X PCR buffer (Perkin Elmer Cetus, Norwalk, CT), 8 μl 10 mM dNTPs mixture, 1 μl 5'-primer (100 pmol/μl), 1 μl 5-' and 3'-primer, 0.5 μl AmpliTaq 5 U/μl (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, Perkin Elmer Cetus) and 20 μl of RT mixture. Sterile, double-distilled H_2O was added to the mixture to bring it up to 100 μl. The mixture was overlaid with mineral oil. The PCR conditions were set up as follows: 95°C for 5 min followed by 95°C for 70 sec, 52°C for 70 sec, 72°C for 70 sec for 40 cycles, and 72°C for 10 min extension time and soaked at 4°C. The PCR reaction was performed in an OmniGene temperature cycler (Hybaid, Middlesex, England).

To assess nested primers to a particular gene the PCR mixture after completion through the PCR cycling was added (10 μl) to 10 μl of 10x PCR buffer, 8 μl of 10 mM dNTPs, 1 μl of 5'-nested primer (100 pmol/μl), 1 μl of 3'-nested primer (100 pmol/μl) and 0.5 μl of AmpliTaq

polymerase (5 U/μl). The volume of the mixture was brought up to 100 μl. The PCR cycling was performed as for the first reaction except the annealing temperature was 55°C. The preparation of PCR mixture for the temperature cycler was carried out in a designated PCR room in a specified laminar flow hood.

The PCR amplification product was detected by electrophoresis on a 2% agarose gel (GIBCO BRL, Grand Island, N.Y.) and visualized by ethidium bromide staining under ultra violet light. An ϕX174RF DNA/*Hae* III fragment DNA ladder (BRL) was used as a size reference marker for all assays.

B. RESULTS

The screening process involved examining 10 established melanoma cell lines (10^6 cells/line) and 39 normal PBL (10^7 cells/blood draw) as controls. In Table 4, the expression of these markers is shown for melanoma cells and PBL. A positive reaction was considered as a visible specific PCR amplification product by gel electrophoresis stained with ethidium bromide.

Table 4

PCR analysis of melanoma marker genes

Marker gene	Melanoma cell lines	PBL
Tyrosinase	9/10	0/39
p97	10/10	0/39
MAGE-3	8/10	0/39
MUC18	10/10	2/39

RNA was extracted from melanoma and PBL and assessed for expression of individual markers by PCR. Data presented as positive cell lines or PBL over total number of specimens assessed. Positive PCR refers to PCR amplification product assessed by gel electrophoresis.

All four markers were transcribed in all the melanoma lines, except for MAGE-3. A melanoma cell line expressing all four markers would produce cDNA PCR products of size; 284 base pairs (bp), 286 bp, 423 bp and 437 bp (tyrosinase, p97, MAGE-3 and MUC18 respectively) as observed after electrophoresis through an agarose gel with ethidium bromide staining and compared with DNA size markers. In one melanoma cell line, tyrosinase expression by PCR was negative; however, when nested PCR for tyrosinase was performed tyrosinase gene expression was detected. There was no detection of melanoma markers in PBL from 39 normal donors, except two donors which were positive for MUC18 gene transcription. These individuals were tested multiple times from separate blood draws and always remained positive for MUC18. This indicated they were not false positive results due to PCR contamination or contamination from normal tissue during blood drawing.

In all assays, MUC18 nested primer PCR was performed; this procedure increased the sensitivity to allow verification and amplification of weak bands produced by PCR with only MUC18 primers. Melanoma cell lines and PBL were tested at least twice to verify specificity. Respective controls in each assay included samples with positive RNA for the gene being assessed, PCR reagents and primers without RNA, human tumor cell lines which were negative for individual gene expression, and β-actin gene expression.

MAGE-1, a gene family member of MAGE-3, also was tested and found to be transcribed in less than 50% of the melanoma cell lines. It was decided not to use this marker for melanoma, since MAGE-3 is more highly expressed in melanomas and MAGE-3 is usually found when MAGE-1 is expressed (Gaugler *et al.*, 1994). Expression

of both genes were not detected in PBL from normal volunteer donors.

EXAMPLE II

Sensitivity of Multiple Melanoma Markers

A. MATERIALS AND METHODS

RNA was isolated and quantitated from melanoma cell lines positive for individual markers. Specific marker PCR analysis was then carried out on serial diluted RNA as described in Example I.

(i) Southern blot analysis

After electrophoresis of PCR amplification products, agarose gels were transferred overnight onto nitrocellulose membrane (Schleicher & Schull, Keene, N.H.) with 20X SSC buffer as previously described. The cDNA was then UV-crosslinked onto the membrane and hybridized overnight with a digoxigenin labelled probe (Morisaki *et al.*, 1992). After hybridization, the membrane was washed in 2X SSC, 0.1% SDS for 10 min. at room temperature and then in 0.1X SSC, 0.1% SDS for 30 min. at 68°C to remove nonspecific binding (Sambrook *et al.*, 1989). Specific binding was detected using anti-digoxigenin, alkaline phosphatase-conjugated antibody as described by the manufactor (Genius Kit; Boehringer Mannheim, Indianapolis, IN). Tyrosinase probes were either prepared, full-length from PCR cDNA products using the outermost PCR oligonucleotide primers, or 2K bp probes were *Eco* R1 digested from plasmids containing the tyrosinase gene sequence (Kwon *et al.*, 1987). All other probes used in Southern blotting, were prepared from PCR cDNA products using the outermost oligonucleotide primers.

Gel electrophoresis and Southern blotting also was performed automatically using the Automated Electrophoresis System, National Genetics, Inc. and U.S. Patent No. 5,279,721. See above.

B. RESULTS

In general, all markers could be detected at picogram levels of RNA by visual examination of gel-electrophoresed PCR amplification products stained with ethidium bromide. RNA from melanoma cell lines were diluted in a series from 1 to 10^{-9} μg and assessed for markers tyrosinase, p97, MUC18, and MAGE-3. Sensitivity varied for individual lines with different levels of gene expression. In general, mRNA for p97, MUC18, and MAGE-3 was detected around 10-100 *pg* by PCR. Tyrosinase mRNA could be detected at 10-100 *fg* by PCR.

Specificity of the amplification products was demonstrated by Southern blotting with respective specific probes (tyrosinase, p97). Sensitivity of the PCR assay could be enhanced 10- to 100-fold using PCR followed by probe blotting. Nested PCR for tyrosinase enhanced detection levels 10-100 fold above PCR for tyrosinase. However, nested PCR for MUC18 enhanced results about 10-fold compared to standard PCR for MUC18.

EXAMPLE III

Detection of Melanoma Cells Mixed With PBL *in vitro*

A model system mimicking circulating melanoma cells in blood was developed. In this assay, system 10^7 normal PBL were mixed with serial dilutions of melanoma cells (10^6 to 1 cell) and assessed by PCR for individual gene markers. The PCR amplification products were then assessed by ethidium bromide staining of gels and by Southern blot analysis. RT-PCR amplification was also

performed on RNA extracted from 10^7 PBL and 10^1 melanoma cells, as controls. Southern blot analysis performed for tyrosinase verified the specificity of the PCR amplification product and demonstrated enhanced sensitivity. Materials and methods were as described in Examples I and II.

Gel electrophoresis or nested primers analysis demonstrated that melanoma cells could be detected at about 1 cell in 10^7 PBL for tyrosinase, p97 and MUC18. PBL controls were negative for individual markers in both gel staining and Southern blot analysis and in both standard and nested PCR. Specific dilutions of melanoma cells, were also analysed, in 50 million PBL and demonstrated that about 1-5 melanoma cells could be detected in 50 million PBL with nested primer tyrosinase PCR followed by probing with tyrosinase cDNA.

To demonstrate the sensitivity and reproducibility of detecting 1 melanoma cell in 10 million PBL, a Poisson distribution analysis was carried out. In 8 of 11 samples, a positive PCR amplification product developed by tyrosinase PCR was detected by gel electrophoresis. The level of detection was enhanced >90% when tyrosinase nested PCR primers or Southern blot analysis with tyrosinase probe was performed.

EXAMPLE IV

Assessment of Circulating Melanoma Cells in Patients Blood

A. MATERIALS AND METHODS

(i) Patients

All melanoma patient with complete documented physical and medical histories were accrued from JWCI. Melanoma patients studied were AJCC (American Joint Committee on Cancer) stage I, II, III and IV. Patients assessed were NED (no evidence of clinical disease), AWD (alive with clinical disease) or EXP (expired during follow-up). The accrual and study of patients was carried out in a double-blind fashion. The patients' disease status was not known to the individual running the PCR assay nor the analyzer of the PCR data. Clinical disease status was documented at the time of blood drawing and again at 8-15 month follow-up period. PCR data results were not known to individuals recording the patient status during the follow-up period.

Fifteen ml of blood was obtained from patients and collected in sodium citrate tubes. All blood was drawn in the John Wayne Cancer Clinic using the same procedure. Blood was drawn after written consent was obtained from the patient. The protocol for the study was approved by the Saint John's Hospital and John Wayne Cancer Institute Human Subjects Committee. Tubes were centrifuged for 20 min at 2000 x g. The buffy coat was carefully removed and diluted in double distilled water. The cells were washed by centrifugation for 5 min. All other materials and methods were performed as described in Example Iand II.

(ii) Protocol

Materials and methods were as described in Examples I and II. PBL from melanoma patients were examined using an optimized PCR assay detection system. The protocol was as follows: PCR assays were performed to detect transcripts of tyrosinase, p97, MAGE-3, and MUC18. All melanoma patients were subjected to all four tests. If the sample was negative for tyrosinase or MUC18, nested PCR was performed with respective primers. If the PBL specimen was negative in the PCR assay for tyrosinase nested primers and p97 markers, then Southern blot analysis would be performed with respective probes. PBLs negative for all the markers and tests were considered as true negatives.

Initially, PBL isolated by Ficoll-hypaque gradient centrifugation were compared to buffy coat isolated PBL. In the analysis, buffy coat isolated cells appeared better for the detection of circulating melanoma cells in blood by PCR.

B. RESULTS

A summary of the analysis of blood specimens by PCR using multiple markers, as assessed by ethidium bromide stained gels, is shown in Table 5. The greatest number of positive patients was observed with MUC18 (73%), with tyrosinase (59%), p97 (54%) and MAGE-3 (10%) identifying few. Analysis with nested primers of tyrosinase versus tyrosinase primers significantly increased the number of positive patients from 2 to 57. Further analysis of p97-negative and tyrosinase-negative patients with respective specific probes significantly increased the number of positive patients. The most significant increase was observed by Southern blotting with the p97 probe. Six

patients were positive for all four markers. All six patients were stage IV.

Table 5

Analysis of melanoma patients using multiple marker PCR assay

	Number of patients positive			
Assays	p97	tyrosinase	MAGE-3	MUC18
PCR	16	2	12	80
Nested PCR	-	57	-	87
cDNA blot	49	12	-	-
Total patients	65	71	12	87

Melanoma patients (120) were evaluated. PCR and cDNA blot refer to assays positive for individual marker genes. Nested PCR refer to specimens tested negative for tyrosinase PCR, and + or - for MUC18 PCR that become positive after nested PCR. cDNA blot refers to patients tested that were negative for either PCR or nested PCR and became positive after cDNA blotting.

The PCR analyses were correlated with disease stage and status (AWD & EXP, NED) of patients. The follow-up time for clinical status after blood drawing for PCR analysis was 8-15 months. In the study, there were 4, 18, 32, and 66 Stage I, II, III, and IV patients, respectively. The majority of the patients in individual Stages II to IV were PCR positive. Table 6.

Table 6

PCR positive patients correlation to Disease status patient status

AJCC Stage	AWD & EXP	NED
I	NP	1/4
II	NP	16/18
III	5/6	23/26
IV	46/48	17/18
Total positive	51/54 (94%)	57/66 (86%)

Values represent patients PCR positive (1 or more markers) over total patients evaluated. NP refers to no patients. AWD & EXP refer to patients AWD and those who were AWD during the blood draw and expired (EXP) during the follow-up period.

The detection of PCR markers was correlated with the Breslow thickness and Clark level of the primary melanoma, after it had been surgically removed. The latter two factors play a role in determining the patients prognosis (Breslow, 1970; Morton *et al.*, 1993). Breslow thickness has been shown to correlate very well with disease progression. Breslow thickness was divided into subgroups of 0.75 mm or less, >0.75 mm to 1.49 mm, ≥1.5 mm to 3.0 mm and >3.0 mm. However, there was no significant correlation of Breslow thickness and detection of PCR markers. Although the majority of the patients were either Clark's level 3 or 4, no significant pattern was observed for Clark's level and number of positive PCR markers. Neither the number of tumor-positive regional lymph nodes nor the sites of distal metastases significantly correlated with the number of positive PCR markers.

The lack of correlation between primary melanoma Breslow thickness and Clark's level with the number of PCR positive markers may be due to the fact that tumor

progression is no longer dependent on these initial pathological parameters of the primary tumor once it has been removed.

EXAMPLE V

Statistical Analysis

To assess the difference between using tyrosinase alone as a marker and using tyrosinase, MUC18, p97 and MAGE-3 together, a coefficient in level for small sample proportion analysis was performed. Assessment of significance of disease stage to PCR data that was analyzed is summarized below:

n = 120	Stage I =	4	NED = 65
	Stage II =	18	AWD = 38
	Stage III =	32	EXP = 17
	Stage IV =	66	

Of the 120 patients, 49 tested negative for tyrosinase. 42 of these tested positive for at least one of the other three markers (MUC18, P97, MAGE-3). This improvement is statistically significant at the 99% confidence level. It can therefore, be concluded that the four marker PCR assay is more sensitive than the single marker (tyrosinase) assay.

Next, an attempt to correlate a patient's disease stage (I, II, III, or IV) and the number of positive markers (0-4) was undertaken. Table 7 shows the breakdown.

Table 7

Number of PCR markers correlated to stage and disease status

	Patients Number of positive markers					
Disease stages	0	1	2	3	4	Total Patients
I	3	0	0	1	0	4
II	2	6	5	5	0	18
III	4	5	15	8	0	32
IV	3	15	23	18	7	66
Total	12	26	43	32	7	120

Positive markers refer to detection of tyrosinase, p97, MUC18 and MAGE-3 by either PCR, nested PCR or Southern blotting.

The results show a positive correlation between stage and the number of positive markers, $p = 0.0025$, *i.e.*, as stage increases, the proportion of positive markers also seems to increase.

In the follow-up period after blood drawing, patients were divided into those with clinical evidence of disease progression and those with no evidence. The number of patients positive for 0 to 4 PCR markers was correlated to disease progression. The relationship between progression and the number of positive markers also was assessed. Analysis showed that there was a significant correlation ($p < 0.05$) between number of positive markers and disease progression. Table 8.

Table 8

Number of PCR markers correlated to progression of disease

	0 pos	1 pos	2 pos	3 pos	4 pos	TOTAL
No Progression	9	13	32	17	2	73
Progression	3	13	11	15	5	47
TOTAL	12	26	43	32	7	120

Thus, although tyrosinase has been used as a marker in a previous report, the studies disclosed herein indicate that tyrosinase alone is not always sensitive in detecting circulating melanoma cells. The use of more than one marker can verify the presence of occult melanoma cells and significantly increase the sensitivity of detecting melanoma cells that express few or no copies of tyrosinase mRNA. The study demonstrated that using four markers was significantly better than tyrosinase alone. In addition, the number of markers detected in individual patients correlated, significantly, with stage and disease progression. This higher expression of individual marker genes indicates, that there is an increase in the heterogeneity of tumor cells or an increase in the number of cells in circulation, at advance stages of disease.

Overall, the level of detection was similar for tyrosinase and p97 markers. MUC18 marker was the most frequently detected whereas, MAGE-3 was the lowest. Although MAGE-3 is expressed in cell lines and biopsies in higher frequency, the number of mRNA copies in a single tumor cell is likely to be very low. This may be related to the state of the cell or clonal phenotype during circulation in the blood.

EXAMPLE VI

Detection of β-HCG mRNA Expression in Melanoma Cells

A. MATERIALS AND METHODS

(i) Melanoma Cell Lines

Twenty-four melanoma cell lines were established and characterized at John Wayne Cancer Institute as previously described (Hoon *et al.*, 1991). Cell lines were cultured and passaged as described in Example I.

(ii) RNA extraction

Total cellular RNA was extracted, isolated and purified using Tri-Reagent according to the manufacturer's protocol (Molecular Research Center, Inc. Cincinnati, OH) and described in detail in Example I. Cells from melanoma lines were lightly trypsinized and collected from tissue culture flasks. Biopsy specimens if cryopreserved were rapidly thawed and kept in a ice water bath. Tumor biopsies were kept in a ice water bath when being minced. All RNA extraction was carried out in a sterile designated laminar flow hood with RNase free labware. Purified RNA was quantitated and assessed for purity by UV spectrophotometry.

(iii) Oligonucleotide primers and Probes

Oligonucleotide primers were synthesized and purified at the Molecular Biology Institute Core Facility, UCLA. The β-HCG primer sequences were as follows: 5' primer was 5'-ATGCCACCCTGGC TGTGGAGAA-3' (SEQ ID NO: 13) and the 3' primer was 5'-GGGAGTCGGGATGGACTTGGAA-3' (SEQ ID NO: 14). The RT-PCR cDNA product was 367 bp. The 5'primer has only one mismatch with the β-luteinizing hormone (LH, see below)

while the 3' primer is homologous to both β-HCG AND β-LH coding regions. A full-length, PCR product, amplified from β-HCG DNA, was used a probe for Southern blot analysis.

The sequences of α-HCG primers were derived from the GenBank; 5'-AAAGGAGCGCCATGGATTAC-3'(SEQ ID NO: 15); and 3' primer, 5'-CCATTACTGTGACCCTGTTA-3'(SEQ ID NO: 16). The α-HCG PCR cDNA product was 297 bp. The primer sequences for β-HCG/LH receptors were 5'primer, 5'-CCCGATGTGCTCCTGAACCAGA-3'(SEQ ID NO: 17); and 3'primer, 5'-GCTGACACCGACAAGGGGCAA-3' (SEQ ID NO: 18). The RT-PCR cDNA product for β-HCG/LH receptors was 496 bp. The β-actin primer sequences were as follows: 5' primer was 5'-GGAGCAATGATCTTGATCTTC-3' (SEQ ID NO: 21) and the 3' primer was 5'-CCTTCCTGGGCATGGAGTCCTG-3' (SEQ ID NO: 22). The RT-PCR product was 201 bp. The tyrosinase and MAGE-3 primers were the same as described in Example I.

(iv) RT-PCR assay

The RT-PCR assay was carried out as previously described (Morisaki *et al.*, 1992, and in Example I). Briefly, reverse transcription was carried out with oligo $(dT)_{15}$ primer and AMV reverse transcriptase with 5 ug of RNA and incubated for 2 hr at 42°C and 99°C for 5 min. The RT-PCR conditions were set up as follows: 95°C for 5 min followed by 95°C for 1 min, 65°C for 1 min, 72°C for 1 min, and 72°C for 10 min for final primer extension sequence and performed in an OmniGene thermocycler (Hybaid, Middlesex, England).

(v) Restriction digestion

β-HCG is a gonadotropin hormone composed of an α- and β-subunit (Giuliano, *et al.*, 1995; Fiddes *et al.*, 1979; Boorstein *et al.*, 1982). The amino acid sequence of α-HCG is essentially indistinguishable from those of the other human gonadotropin hormones, such as follicle-stimulating, luteinizing, and thyroid-stimulating hormones (Fiddes *et al.*, 1979; Pierce *et al.* 1981). However, the β-HCG subunit is different amongst the other hormone subunits except for the β-LH subunit; they share 82% common amino acid sequence (Talmadge *et al.*, 1984). The ß-subunit of HCG to date has been shown to consist of cluster of 6 related genes linked closely to the ß-LH single gene (Bo *et al.*, 1992). Since the β-HCG and β-LH are highly homologous it is not possible to design a primer sequence absolutely specific to β-HCG.

However, the β-HCG PCR cDNA product has a unique *Sty* I restriction site that is not present in the β-LH PCR cDNA product. Digestion of PCR products with this enzyme allows β-HCG to be destinguished from β-LH. RT-PCR cDNA product was incubated with 10X NEBuffer 3 (New England BioLabs, Beverly, MA) and *Sty* I (10 U/ml) (New England Biolabs) and the mixture was incubated overnight at 37°C. The endonuclease digested product mixture was run on a 2% agarose gel and stained with Etbr. β-HCG RT-PCR cDNA product digested with *Sty* I produces a 271 and 96 bp band. If no digestion occurred the reaction was repeated at least twice to confirm.

(vi) Southern blotting

RT-PCR cDNA products run on a 2% agarose gel were denatured and transferred overnight onto nylon membrane (Micron Separations, Inc.) as previously described in Example II. β-HCG cDNA probe was prepared by PCR,

purified and digoxigenin labelled as described in Example II.

B. RESULTS

Assessment of β-HCG expression in cells by molecular techniques has been difficult because of the sequence homologies of both α and β subunits to related hormones. The terminal end of the β-chain subunit chain was chosen as a target for RT-PCR since it had the most significant differences compared to other related hormone β-chain subunits.

Initially 24 established human melanoma cell lines derived from different anatomical sites were assessed to determine if they expressed β-HCG chain. Oligo $dT_{(15)}$ priming was carried out to assess only poly A mRNA of β-HCG. Of the 24 cell lines tested by RT-PCR, 16 of 24 produced a specific cDNA product of the correct size (367 bp) as verified by Etbr gel staining.

β-actin was run on all samples as an internal control to verify RNA yield and efficiency of the RT-PCR assay. Each assay had a negative control consisting of RT-PCR reagents alone without RNA and a positive control for β-HCG. Southern blot analysis of PCR cDNA product with the β-HCG probe showed that three of the cell lines negative by Etbr staining had a specific cDNA band. However, one cell line in which the Etbr staining was questionable showed no specific band on Southern blot analysis. Overall 18 out of 24 cell lines were positive (75%) for β-HCG marker expression.

To further verify β-HCG marker expression, endonuclease restriction digestion with *Sty I* was carried out on the RT-PCR cDNA products. All cDNA products digested, produced two bands, 271 bp and 96 bp as

observed on Etbr gels, indicative of β-HCG marker. These digested products were further verified by Southern blot analysis with β-HCG cDNA probe.

EXAMPLE VII

Detection of β-HCG mRNA Expression in Melanoma Tumor Biopsy Specimens

A. MATERIALS AND METHODS

(i) Melanoma Tumor Biopsy Specimens and Blood Preparation

Melanoma tumor biopsy specimens that were defined by histopathology as malignant melanoma were assessed. Melanoma biopsies were obtained from primary lesions and from multiple anatomical sites of metastatic lesions from different patients. Specimens were immediately frozen or processed as received from the operating room. In this study liquid nitrogen cryopreserved and fresh tumor biopsies from surgery were assessed. On obtaining melanoma biopsies non-melanoma tissue was carefully dissected away from normal tissue under sterile conditions in a laminar flow hood.

PBL were obtained from 25 normal male and female volunteer donors and the buffy coat was collected for RNA isolation as described in Example I. All other techniques including RT-PCR, Southern blotting and restriction enzyme digests were as described in Example VI.

Normal axillary lymph node tissue that was assessed as histopathology negative for tumor was obtained from melanoma and breast cancer patients undergoing elective surgery. Axillary lymph nodes were assessed by

histopathology for malignancy by standard conventional hematoxylin and eosin (H & E) staining.

B. RESULTS

Melanoma primaries and metastases have been shown to contain infiltrating immune cells (Cochran and Hoon, 1987). To be certain that β-HCG mRNA was being expressed by the tumor cells and not a product of infiltrating lymphoid cells, peripheral blood lymphocytes were also analysed for expression of β-HCG mRNA. PBL from 25 normal volunteer donors were analysed by RT-PCR but no evidence of β-HCG expression was observed, even after Southern blot analysis (except in one individual who was positive in a second blood draw).

Five lymph nodes from two breast and melanoma patients, were found to be negative by H & E staining and by RT-PCR and Southern blotting.

Both cryopreserved and fresh biopsy tissue were anaylsed by histopathology and by RT-PCR, restriction digestion and Southern blotting. Out of 40 patients, 38 were identified, as melanoma positive by histopathology, while 16 were identified as positive by RT-PCR for β-HCG marker. In other words, an estimated 42% of melanoma biopsies were β-HCG positive. All specimens that were found to be melanoma negative by histopathology, were also negative for β-HCG marker expression. The detection of β-HCG mRNA was much weaker in melanoma biopsy tissues as compared to melanoma cell lines. This detected lower gene activity may be due to the heterogeneity of tumors, variability of host physiologic regulation of β-HCG, or simply the dilution of RNA by normal cell infiltrate.

There was no significant difference in β-HCG mRNA detection between cryopreserved and fresh biopsy

specimens. β-actin expression was detected in all specimens, thus verifying the integrity of the mRNA and the PCR assay. α-HCG subunit expression in five β-HCG positive melanoma cell lines and five melanoma biopsies was also analyzed. However, no α-HCG expression was detected by RT-PCR even when followed by Southern blot analysis.

EXAMPLE VIII

A Comparison of β-HCG mRNA Expression with Other Melanoma Markers

A. MATERIALS AND METHODS

(i) Surgical Specimens

Axillary lymph node tissue was taken after elective surgery of TDLN from seven melanoma patients. TDLN were assessed by histopathology for malignancy by standard conventional hematoxylin and eosin (H & E) staining. β-HCG mRNA expression was compared with tyrosinase and MAGE-3 mRNA expression by RT-PCR. All other materials and methods were as described in Example VI.

B. RESULTS

Out of eight tumor-draining lymph nodes (TDLN) (from seven melanoma patients) five were positive for β-HCG expression, six for tyrosinase and three for MAGE-3. In two patients, none of the markers were detected.

Table 9

Analysis of β-HCG expression in melanoma TDLN

TDLN	Path	β-actin	β-HCG EtBr	β-HCG S. Blot	β-HCG Sty I	Tyr	MAGE3
1	+	+	-	+	+	+	-
2	+	+	-	+	+	+	-
3	+	+	-	-		+	+
4	+	+	+	+	+	+	+
5	+	+	-	-		-	-
6	+	+	+	+	+	+	+
7a	+	+	+	+	+	+	-
7b	-	+	-	-		-	-

TDLN refer to individual patient nodes examined (a and b refer to two separate nodes). Pathology refers to the hematoxylin and eosin staining histopathology analysis of lymph node sections. + refers to presence of melanoma metastases and - refers to no metastases. RT-PCR analysis detected by Etbr and Southern blot is indicated as + or -. Tyr refers to RT-PCR analysis by tyrosinase primers followed by nested tyrosinase RT-PCR if negative.

In conclusion, β-HCG is a useful addition to the group of melanoma markers described in Examples I through V. The frequency of expression of β-HCG mRNA in melanoma appears to be similar to that of the melanoma tumor antigens MAGE-3 and MAGE-1.

EXAMPLE IX

Detection of GalNAc mRNA Expression in Melanoma Cells and Biopsies

A. MATERIALS AND METHODS

(i) Melanoma cell lines and *Surgical Specimens*

Melanoma cell lines were all established at JWCI and grown as described in Example I. 20 melanoma tumor

biopsy specimens were obtained as described in Example VII. RNA extraction and RT-PCR assay was as described in Example BI.

(iv) Oligonucleotide primers and Probes

Oligonucleotide primers were synthesized and purified at the Molecular Biology Institute Core Facility, UCLA. The GalNAc primers used were: 5'-CCAACTCAACAGGCAACTAC-3' (SEQ ID NO: 19) and 3' GATCATAACGGAGGAAGGTC-3' (SEQ ID NO: 20). cDNA probes, amplified by PCR with these primers, were used in Southern blotting, which was performed as described in Example II. The tyrosinase and MAGE-3 primers were the same as described in Example I and β-HCG primers were the same as described in Example VI.

Table 10

GalNAc expression in melanoma biopsies and cell lines

SPECIMENS	GalNAc EXPRESSION		
BIOPSIES# METASTASES			
19	-		
25	-		
68	-		
100	+		
102	+		
178	-		
221	-		
224	-		
246	+		
250	-		
260	-		
261	-		
287	-		
292	+		
295	+		
301	+		
351	+		
361	+		
380	+		
443	+		
MELANOMA CELL LINES			
MATT	+		
M101	+		
M12	+		
M24	+		
M10	+		
M18	+		
MKN	+		

Table 10 (continued)

SPECIMENS	GalNAc EXPRESSION		
MHL	+		
MCE	+		
MKE	+		
MELL	+		
MMAC	+		
MF	-		
M18	+		
PERIPHERAL BLOOD LYMPOCYTES			
DONOR #1	-		
DONOR #10	-		
DONOR #12	-		
DONOR #13	-		
DONOR #322	-		
DONOR #323	-		
DONOR #324	-		
DONOR #325	-		
DONOR #326	-		
DONOR #338	-		
DONOR #339	-		
DONOR #340	-		
DONOR #342	-		
DONOR #343	-		
NORMAL LYMPH NODE #349	-		
NORMAL LYMPH NODE #364	-		

B. RESULTS

As shown in Table 7, detection of GalNAC mRNA was succesfully detected in 13 out of 14 melanoma cell lines and 10 out of 20 biopsy specimens. Furthermore, no GalNAc marker expression was observed in normal lymph nodes or PBL. These are similar results to those found for β-HCG and MAGE-3 in previous examples. Indicating that GalNAc mRNA expression is another marker which may be utilized for the detection of melanoma and metastases.

Amplification of GalNAc mRNA is an indicator of gangliosides, GM2 and GD2, expression. Direct detection of GM2 and GD2 in occult metastases and small tumor lesions such as melanoma primaries is very difficult and often impractical when using standard biochemical methods. Monoclonal antibodies to gangliosides are available but often cross-react with other carbohydrate structures and therefore are not reliable and do not represent absolute ganglioside expression (Hoon *et al.*, 1993).

Detection of tumor cells with the marker GalNAc by RT-PCR provides a novel approach to detect metastatic melanoma and breast cancer cells in blood or fluids that would not be possible by current biochemical or immunological techniques.

EXAMPLE X

Detection of β-HCG mRNA Expression in Breast Cancer Cells

A. MATERIALS AND METHODS

(i) Breast cell lines

The established breast cancer cell line JWCI BM-1 was developed from a primary invasive ductal carcinoma

tumor and characterized as a breast cancer line at the John Wayne Cancer Institute. Breast cell lines MDA-MB-231, MCF-7, BT-549, T-47D and BT-20 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured according to instructions provided. The 734B line is an established subclone of MCF-7. Cells were grown in 10% fetal calf serum (heat-inactivated) RPMI 1640 (Gemini Bioproducts, Calabasas, CA) plus penicillin and Streptomycin (GIBCO, Long Island, NY) in T75 cm^2 flasks. Adherent cell lines were routinely passaged by trypsinization every 3-4 days. When cell lines attained 75-85% confluency they were used for PCR analysis.

(ii) RNA preparation

Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH) was used to isolate total RNA from the cell lines and surgical specimens, following the manufacturer's instructions and described in Example I. One μg of total RNA was used in the PCR assay to detect β-HCG mRNA. Oligonucleotide primers and probes were as described in Example VI.

(iii) RT-PCR

Reverse transcription was as described in Example I, using oligo $(dT)_{15}$ and oligo nucleotides as described in Example VI.

The PCR mixture was also as described in Example I and incubated in an OmniGene temperature cycler (Hybaid, Middlesex, England) at 95°C for 3 min for 1 cycle; 95° C for 1 min, 65°C for 1 min, 72°C for 1 min for 30 cycles; and 72°C for 10 min. The PCR cDNA products were assessed in a 2% agarose gel containing ethidium bromide. A 100 bp DNA ladder (GIBCO BRL Life Technologies Inc.,

Gaithersburg, MD) was used as a bp reference marker. Restriction enzyme digestion and Southern blot analysis were performed as previously described in Example VI.

(iv) β-HCG protein expression in breast cancer cells

Breast cancer cells from individual cell lines were seeded in 12 well tissue culture microplates at 2 million cells/well in 1.5 ml of RPMI 1640 without fetal calf serum and cultured at 37°C in a tissue culture incubator for 24 hr. Supernatant was harvested and concentrated 10 times to a volume of 150 μl using Centricon 10 concentrators (Amicon Division, W.R. Grace & Co., Beverly, MA). β-HCG in the supernatant was measured using a total β-HCG Quantitative Test kit (Medix Biotech Inc., Foster City, CA), following the manufacturer's instructions. Supernatant samples were tested in duplicate with a standard reference for each assay. The ELISA reaction was read at 490 nm using a Vmax kinetic microplate reader (Molecular Devices Corp., Palo Alto, CA).

B. RESULTS

(i) β-HCG mRNA expression in breast cell lines

All seven breast cancer cell lines were found to express β-HCG mRNA. A positive result was indicated by a 367 bp cDNA band as detected by Etbr-staining and Southern blot analysis. To confirm the identity of the amplified PCR cDNA product, samples were digested with endonuclease *Sty I*. All seven β-HCG PCR products were cleaved by *Sty I* to produce bands of 271 and 96 bp on Etbr gels, thus confirming β-HCG mRNA expression. As a negative control, PBL from 25 normal (male and female) volunteers were examined. None of the control specimens

were shown to be positive for β-HCG marker expression by PCR and Southern blotting.

The expression of α-HCG mRNA was also examined by PCR in breast cell lines. In cell lines MDA-MB-231, JWCI BM-1, and T-47D, α-HCG mRNA expression was detected by Etbr-stained gel electrophoresis. The α-subunit detected could be HCG or other related hormones since they all share a high degree of similarity in α-subunit. For this reason α-HCG as a cancer marker, is not practical.

(ii) β-HCG protein expression in breast cancer cell lines

Out of the seven breast cell lines expressing β-HCG mRNA, only three cell lines (MDA-MB-231, T47-D, JWCI BM-1) secreted detectable levels of β-HCG protein as analysed by ELISA (0.15 mIU, 0.15 mIU, 0.1 mIU/2 x 10^6 cells, respectively). The cell lines, producing β-HCG were those that were positive for α-HCG mRNA expression.

Breast cancer lines were also analysed for β-HCG receptor mRNA. Human β-HCG/LH receptor genes cloned recently, have been shown to possess a high dgree of similarity (Minegish *et al.*, 1990). All cell lines were positive for β-HCG/LH receptor mRNA expression. To evaluate whether β-HCG/LH receptor could be used as a marker for metastatic breast cancers, PBL from six normal volunteer donors (male and female) was analysed for the corresponding mRNA using the primers as described in Example VI. All donors expressed β-HCG/LH receptor mRNA indicating that the β-HCG/LH receptor is not a reliable marker for detecting breast cancer cells in blood or lymph nodes.

EXAMPLE XI

Sensitivity of β-HCG Marker

A. MATERIALS AND METHODS

(i) PCR detection sensitivity

The sensitivity of the PCR assay to detect β-HCG mRNA in breast cancer cells was assessed by the following methods:

(a) RNA was isolated from MDA-MB-231 cells and serially diluted from 1 to 10^{-6} μg, and then analysed by the RT-PCR assay.

(b) Cell suspension of MDA-MB-231 cells were prepared and diluted with PBL to produce an *in vitro* model occult carcinoma cells in lymph nodes. 10^7 PBL were mixed with a variable number of cancer cells ranging from 1 to 10^5. Total RNA was then isolated from the mixtures and analysed by the RT-PCR assay and observed on an Etbr gel along side a positive (10^6 MDA-MB-231 cells) and negative (10^7 PBL) control. All other method were as described in Example X. The PBL were obtained from normal volunteers in which PCR analysis had shown no presence of β-HCG mRNA.

B. RESULTS

RT-PCR results of a series of diluted RNA isolated from MDA-MB-231 was determined on Etbr agarose gels. β-HCG marker was detected from as little as 10^{-5} ug RNA. This detection was enhanced ten fold by Southern blotting, enabling β-HCG mRNA expression detection from as little as 10^{-6} μg of RNA.

Employing the *in vitro* model it was shown that one breast cancer cell, determined by the amplification of β-HCG marker, could be detected up to among 10^7 PBL.

EXAMPLE XII

Detection of β-HCG mRNA Expression in Breast Cancer Specimens

A. MATERIALS AND METHODS

(i) Surgical Specimens

Thirty-one lymph nodes were collected from 18 patients (13 invasive ductal carcinoma, 4 invasive lobular carcinoma, and one *in situ* carcinoma) who were undergoing mastectomy with axillary lymphadenectomy for clinically early stage breast cancer. Nodes that were only logistically practical for cutting, for conventional pathological diagnosis and for archive fixation were obtained for RT-PCR. Patients ranged in age from 37 to 73 years old. In order to compare the results of PCR with histological analysis, the lymph nodes were divided into two, one half was analyzed by PCR and the other by histopathological H & E staining of serial sections.

For a negative control, blood was obtained from 25 normal volunteer donors (both male and female). All further materials and methods were as described in previous examples.

B. RESULTS

Table 11 shows the results of β-HCG mRNA expression in TDLN. Two TDLN from eleven patients (patient G to Q) and three TDLN from one patient were analysed. Five of the TDLNs were found to be negative by conventional H & E

staining, were found to be positive for β-HCG marker expression in the PCR assay (No. 4, 16, 19, 23, and 24). If the RT-PCR assays were found to be negative, Southern blot analysis was subsequently performed.

Overall, 9 out of 31 TDLN were found to be negative by H & E staining and PCR with or without Southern blotting. Four of the TDLN which were found to be negative by both PCR and histological examination, were subsequently found to be positive following Southern blot analysis (No. 12, 13, 15, and 22). All 367 bp β-HCG PCR cDNA products detected by PCR or Southern blotting were digested by restriction enzyme *Sty* I. There were no TDLN found to be positive by histological examination, but found negative by PCR or PCR and by Southern blotting.

Table 11

Detection for β-HCG expressing tumor cells in breast TDLN

Specimen number	Patient	Tumor histology	Pathology		PCR	Southern blot
1	A	lobular	+	+		
2	B	ductal	+	+		
3	C	ductal	+	+		
4	D	insitu	-	+		
5	E	lobular	+	+		
6	F	ductal	+	+		
7	G	ductal	-	-		-
8			-	-		-
9	H	ductal	-	-		-
10			+		+	
11	I	ductal	-	-		-
12			-	-		+
13	J	ductal	-	-		+
14			-	-		-
15	K	ductal	-	-		+
16			-	-		+
17	L	ductal	-	-		-
18			+	+		+
19	M	ductal	-	+		
20			+	+		
21	N	ductal	-	-		-
22			-	-		+
23	O	ductal	-	+		
24			-	+		
25	P	ductal	+	+		
26			+	+		
27	Q	ductal	-	-		-
28			-	-		-
29	R	lobular	+	+		

Table 11 (continued)

Specimen number	Patient	Tumor histology	Pathology	PCR	Southern blot
30			+	+	
31			+	+	

Individual patients are labelled as A - R and numbers refer to individual TDLN. Tumor histology represents H & E staining diagnosed pathology. Pathology refers to diagnoses of + or - for breast cancer metastases. PCR results are indicated as + or - on Etbr gel electrophoresis analysis. Specimens negative for RT-PCR assay were subsequently Southern blotted with β-HCG cDNA probe. Southern blot analysis is indicated as + or -.

EXAMPLE XIII

Detection of Breast Cancer Cells by Multiple Markers

A. MATERIALS AND METHODS

(i) Breast cell lines and Surgical Specimens

The breast cell line MDA-MB-231, MCF-7, BT-549, T-47D and BT-20 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured according to instructions provided. The 734B line is an established subclone of MCF-7. Cells were grown as described in Example X. 11 breast cancer biopsies were extracted as described in Example XII. RNA extraction and RT-PCR assay were as described in Example X.

(ii) Oligonucleotide primers and Probes

Oligonucleotide primers were synthesized and purified at the Molecular Biology Institute Core Facility, UCLA. The MAGE-1 primer sequences were as follows: 5' primer was 5'-GCTGGAACCCTCACTGGGTTGCC-3' (SEQ ID NO: 23) and the 3' primer was

5'-CGGCCGAAGGAACCTGACCCAG-3'(SEQ ID NO: 24). The RT-PCR cDNA product was 421 bp. The tyrosinase and MAGE-3 primers were the same as described in Example I, β-HCG primers were the same as described in Example VI and GalNAc primers were the same as described in Example IX. cDNA probes, amplified by PCR with these primers, were used in Southern blotting, which was performed as described in Example II.

B. RESULTS

Multiple markers were used to analyse breast cancer cells and breast cancer biopsy specimens by RT-PCR and Southern blotting. Tables 12 and Table 13 shows the results. All breast cancer cells were positive for at least five, out of the six, markers. For the biopsy specimens, at least one of the markers were detected from all samples. None of the markers alone would have been able to detect cancer cells in all of the specimens. This variation in marker detection reflects the heterogeniety of tumor cells. In conclusion, multiple markers are more sensitive to detection of breast cancer than any one marker.

Table 12

Analysis of Markers in Breast Cancer Cell Lines

	MAGE3	*MAGE 1*	*MUC18*	*p97*	*GalNAc*	*β-HCG*
BT20	+	+	+	--	+	+
BT549	+	--	+	+	+	+
902P	+	+	+	+	+	+
T47D	+	+	+	--	+	+
734B/24	+	+	+	+	+	+
231/45	--	+	+	+	+	+
MCF7	+	+	+	+	+	+

+/-: RT-PCR or Southern Blotting

Table 13

Analysis of Markers in Breast Cancer Biopsies

Biopsy tumor or number	GalNAc	β-HCG	MUC 18	P-97	MAGE-3	MAGE-1
0350T2A122794	+	+	+	--	--	+
0424T2A011795	--	--	--	--	+	--
0433T2A011895	--	+	+	--	+	--
044T2A012095	--	--	--	--	+	--
0460T2A012695	--	+	+	--	+	+
0498T2K020395	--	--	+	--	--	--
0500T2K020695	--	--	+	+	+	--
0506T2A020795	+	+	+	+	--	+
0520T2A020995	--	--	+	--	--	+
0522T2A020995	--	--	+	--	--	--
0525T2A020995	--	--	+	+	--	--

+/-: RT-PCR and Southern Blotting

Previous PCR studies have not analyzed large numbers of patients with different clinical stages of melanoma or breast cancer. This is important in evaluating the sensitivity and clinical significance of the assay. Furthermore, this information is useful in staging disease into clinical subgroups, in particular, identifying subgroups of patients that need more intensive therapeutic intervention. For example, in NED patients with circulating tumor cells, immediate therapeutic intervention may be a very efficacious means of controlling potential tumor progression and, thus, preventing clinical disease. The detection of circulating cancer cells may also prove useful for monitoring a patient's response to operative and adjuvant therapies.

Applying a multiple melanoma marker method to the evaluation of circulating cancer cells also provides information about the tumor's phenotype. Identification of specific tumor-associated antigen(s) permits the rational use of specific immunotherapy protocols such as monoclonal antibodies and cancer vaccine (Hoon *et al.*, 1993). The PCR assay also provides a rapid monitoring system as a follow-up to determine if a specific therapy is effective.

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: John Wayne Cancer Institue
(B) STREET: 2200 Santa Monica Boulevard
(C) CITY: Santa Monica
(D) STATE: California
(E) COUNTRY: United States of America
(F) POSTAL CODE (ZIP): 90404

(A) NAME: National Genetics Institute
(B) STREET: 5839 Green Valley Circle, Suite 104
(C) CITY: Culver City
(D) STATE: California
(E) COUNTRY: United States of America
(F) POSTAL CODE (ZIP): 90230

(ii) TITLE OF INVENTION: DETECTION OF MELANOMA OR BREAST METASTASES WITH A MULTIPLE MARKER ASSAY

(iii) NUMBER OF SEQUENCES: 24

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: UNKNOWN

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: USSN 08/406,307
(B) FILING DATE: 17-MAR-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAAGCCGGCC CAGGCTCG 18

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGAGTCCTCA TAGGATTGGC TCC 23

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCAAGGCAAC CTCAGCCATG TC 22

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CTCGACTCCA CAGTCTGGGA CGACT 25

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTCATCTTCC GTGTGCGCCA 20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GTAGCGACCT CCTCAGGCTC CTTAC 25

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TTGGCAGATT GTCTGTAGCC 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AGGCATTGTG CATGCTGCTT 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GTCTTTATGC AATGGAACGC 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCTATCCCAG TAAGTGGACT 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TACCTGGTGG AGAGCGGCCG CCTC 24

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AGCGTCTTCC CCATCAGTGT 20

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ATGCCACCCT GGCTGTGGAG AA 22

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GGGAGTCGGG ATGGACTTGG AA 22

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

AAAGGAGCGC CATGGATTAC 20

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCATTACTGT GACCCTGTTA 20

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CCCGATGTGC TCCTGAACCA GA 22

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GCTGACACCG ACAAGGGGCA A 21

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CCAACTCAAC AGGCAACTAC 20

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GATCATAACG GAGGAAGGTC 20

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GGAGCAATGA TCTTGATCTT C 21

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CCTTCCTGGG CATGGAGTCC TG 22

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GCTGGAACCC TCACTGGGTT GCC 23

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CGGCCGAAGG AACCTGACCC AG 22

CLAIMS:

1. A method for detecting melanoma or breast cancer cells in a biological sample comprising the step of amplifying at least two nucleic acids in said sample, said nucleic acids being melanoma or breast cancer markers.

2. The method of claim 1, comprising the steps of:

(a) extracting RNA from said sample;

(b) contacting said RNA with a primer pair that hybridize to one of said melanoma or breast cancer marker nucleic acids;

(c) amplifying said melanoma or breast cancer marker nucleic acids to produce an amplification product;

(d) detecting said amplification product; and

(e) repeating steps (b), (c), (d) and (e) with a primer pair that hybridize to at least one of the other said melanoma or breast cancer marker nucleic acids.

3. The method of claim 2, further comprising the step of preparing at least two pairs of primers complementary to regions of said melanoma or breast cancer marker nucleic acids.

4. The method of claim 3, wherein primer pairs for at least three melanoma or breast cancer markers are employed.

5. The method of claim 4, wherein primer pairs for at least four melanoma or breast cancer cell markers are employed.

6. The method of claim 5, wherein primer pairs for at least five melanoma or breast cancer markers are employed.

7. The method of claim 6, wherein primer pairs for at least six melanoma or breast cancer cell markers are employed.

8. The method of claim 7, wherein primer pairs for at least seven melanoma or breast cancer cell markers are employed.

9. The method of claim 2, wherein said melanoma or breast cancer cell markers are selected from the group comprising; tyrosinase, MAGE-3, MUC18, p97, MAGE-1, GalNAc and β-HCG.

10. The method of claim 2, wherein said amplification is polymerase chain reaction.

11. The method of claim 10, wherein said polymerase chain reaction is nested.

12. The method of claim 2, wherein said RNA is total cellular RNA.

13. The method of claim 12, further comprising the step of converting said RNA to cDNA.

14. The method of claim 1, wherein said sample is comprised of a body tissue or body fluid.

15. The method of claim 14, wherein said body fluid, comprising: peripheral blood, lymph fluid, ascites, serous fluid, pleural effusion, sputum, cerebrospinal fluid, lacrimal fluid, stool or urine.

16. The method of claim 14, wherein said body tissue, comprising bone marrow aspirate, bone marrow biopsy, lymph node aspirate, lymph node biopsy, spleen tissue, fine needle aspirate, skin biopsy or organ tissue biopsy.

17. The method of claim 2, wherein said detecting is by gel electrophoresis.

18. The method of claim 2, wherein said detecting is by chromatography.

19. The method of claim 17, further comprising the step of Southern blotting.

20. The method of claim 1, wherein said sample is of human origin.

21. A kit for use in detecting melanoma or breast cancer cell cells in a biological sample comprising:

(a) pairs of primers for amplifying nucleic acids corresponding to the genes for tyrosinase, MAGE-3, MUC18, p97, MAGE-1, GalNAc and ß-HCG; and

(b) containers for each of said primers.

22. The kit of claim 21, further comprising enzymes and reagents for the preparation of cDNA's.

23. The kit of claim 21, further comprising enzymes and reagents for radiochemical or chromophoric labeling of nucleic acids.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC1/US 96/03442

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 00603 (THE TRUSTEES OF PRINCETON UNIVERSITY) 6 January 1994 see page 10, line 26 - page 11, line 8 ---	1-3, 10-20
X	AMERICAN JOURNAL OF CLINICAL PATHOLOGY, vol. 94, no. 4, October 1990, page 507 XP000576592 COX C ET AL: "tumor marker sensitivity single versus multiple markers in patients with breast carcinoma" see abstract 77 ---	1-3, 10-20
X	ISREAL JOURNAL OF MEDICAL SCIENCES, vol. 17, no. 9-10, September 1981, pages 865-8, XP000576568 SULITZEANU D: "markers in breast cancer" see the whole document --- -/--	1-3, 10-23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
18 July 1996	29.07.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

Intern nal Application No
PC1/US 96/03442

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	THE LANCET, vol. 338, 16 November 1991, pages 1227-9, XP002008751 SMITH B. ET AL: "detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction" see the whole document ---	1,23
A	EP,A,0 520 794 (F. HOFFMANN LA-ROCHE AG) 30 December 1992 see the whole document ---	1,23
A	WO,A,90 09456 (BALAZS) 23 August 1990 see claims 1-13 -----	1-3

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.ormation on patent family members

Intern nal Application No
PC1/US 96/03442

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